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TITLE: Induction of Ephs/Ephrins-Mediated Tumor Cells-Endothelial Cells Repulsion
as an Anti-Cancer Therapeutic Approach

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14. ABSTRACT <p>Eph receptors constitute the largest family of receptor tyrosine kinases. They comprise 8 type A receptors and 6 type B receptors. Originally identified as neuronal pathfinding molecules, where they guide the migrating cells to specific tissue targets, they are an essential component in vascular assembly regulation, including during angiogenesis, and in embryonic development. Ephs interact with membrane-bound ligands, the Ephrins, of which 8 have been found to date (Ephrin-A1 to 5 and Ephrin-B1 to 3).</p> <p>We hypothesize that manipulating Eph/Ephrin interactions could prevent attraction and/or induce repulsion between tumor cells and endothelial cells. The goal is to take advantage of Ephs/Ephrins interactions to turn endothelial cells into a barrier to metastatic tumor cells entering the blood flow through vascular endothelial cells, or exiting the flow through bone marrow endothelial cells in the direction of their specific metastatic sites. To test our hypothesis, we first performed a profiling of breast cancer cells with regard to the expression and activation levels of various Ephs/Ephrins. We found that the EphB2 receptor is overexpressed in almost 50 % of the cancer cell lines. Similarly, the Ephrin-B2, which is a ligand to EphB2, is also strongly expressed in over 50 % of the cancer cell lines while not found in benign cell lines. Another EphB2 ligand, Ephrin-B1, is present in all normal cells but lost in 4 out of 11 cancer cell lines. We also found that EphB2 could be subject to defects in phosphorylation and response to the ligand. We are investigating attractive/repulsive behaviour of tumor cells, with different expression levels of EphB2 and EphrinBs, towards endothelial cells and vice-versa. Next, we will study the invasive and metastatic potential of tumor cells with different expression levels of Ephs/Ephrins in vivo.</p>					
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INDUCTION OF EPHS/EPHRINS-MEDIATED TUMOR CELLS-ENDOTHELIAL CELLS REPULSION AS AN ANTI-CANCER THERAPEUTIC APPROACH.

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INTRODUCTION

Eph receptors constitute the largest family of receptor tyrosine kinases. They comprise 8 type A receptors and 6 type B receptors. Originally identified as neuronal pathfinding molecules, where they guide the migrating cells to specific tissue targets, they are an essential component in vascular assembly regulation, including during angiogenesis, and in embryonic development. Ephs interact with membrane-bound ligands, the Ephrins, of which 8 have been found to date (Ephrin-A1 to 5 and Ephrin-B1 to 3). Eph/Ephrin interaction induces a bidirectional signaling in both Eph-bearing and Ephrin-bearing cells and results in either attraction or repulsion between the two partners of the interaction.

Specific interactions between Ephs and Ephrins are involved in restricting intermingling between different cell populations by inducing either cell-cell attraction or repulsion. We hypothesize that manipulating Eph/Ephrin interactions could prevent attraction and/or induce repulsion between tumor cells and endothelial cells.

RESULTS

Task 1. Ephs/Ephrins profiling of breast cancer cell lines.

We aimed at comparing the levels of expression of different members of the Eph/Ephrins family in cancer *versus* normal human mammary epithelial cells. Based on our conclusion that cell density affects expression levels, we have spent some time uniformizing the growth conditions of different cell lines in order to have an expression pattern that is reliable. Also, because of the nonavailability of good antibodies for certain proteins, a major task was the optimization of the western blot experiments for these antibodies.

As shown in figure 1, we observed a differential expression of the Ephs/Ephrins. In particular, using an antibody directed at the N-terminus of EphB2, we found that this receptor is overexpressed in almost 50 % of the cancer cell lines. Similarly, the Ephrin-B2, which is a ligand to EphB2, is also strongly expressed in over 50 % of the cancer cell lines while not found in benign cell lines.

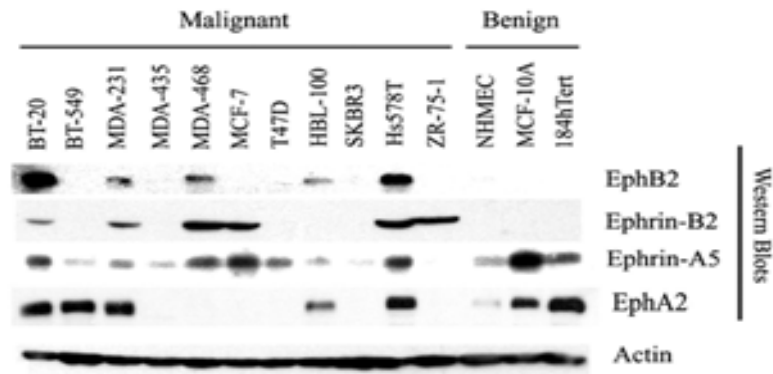


FIGURE 1. Western blot profiling of a panel of human breast cancer cell lines and non-cancer cell lines, checked for the levels of expression of various ephrin ligands and receptors. Actin staining is used as a loading control.

We also performed RT-PCRs on RNA extracted from our panel of cell lines. Unlike the Ephrin-B2 ligand, we found that another EphB2 ligand, Ephrin-B1, is present in all normal cells but lost in 4 out of 11 cancer cell lines (Figure 2). Other Ephrins and Eph receptors did not show a particular pattern of expression.

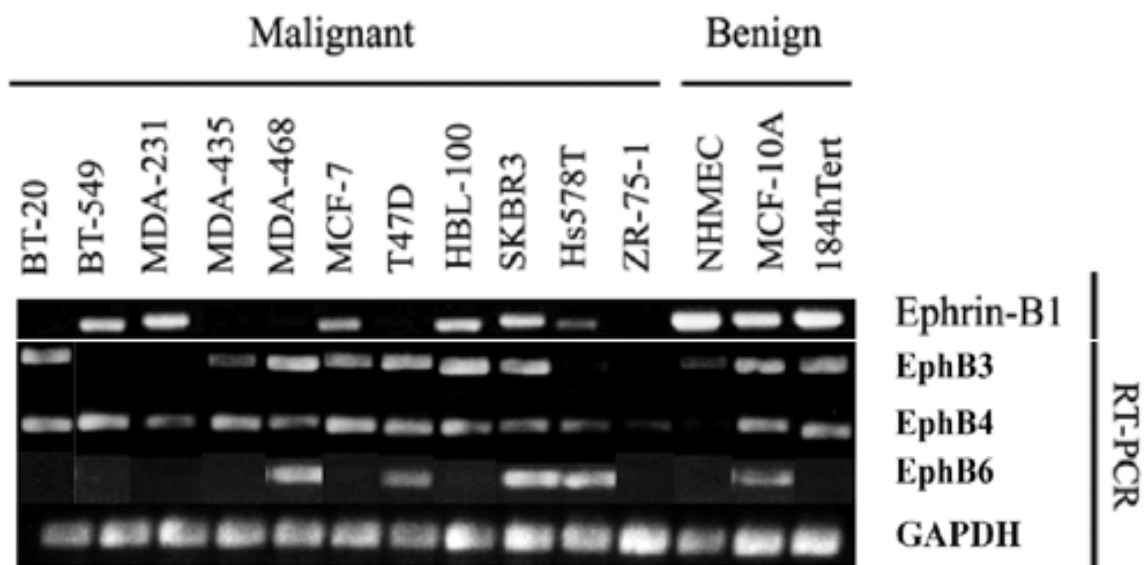


FIGURE 2. RT-PCRs using RNA extracted from different breast cancer and benign cell lines. Amplification of GAPDH was used as a control for RNA amounts.

We next focused on the EphB2 receptor. As part of the characterization of this receptor in breast cancers, we first checked whether it retains its phosphorylation ability following ligand binding, we treated different cell lines with a soluble form of the Ephrin-B1 ligand. As shown in figure 3, while EphB2 phosphorylation is strongly induced in MCF-7 cells which have low levels of EphB2, and in high expressing MDA-231 cells, EphB2 was only moderately phosphorylated in high expressing Hs578T cells.

This indicates that this receptor can be subjected not only to overexpression in breast cancer cells, it could also be subject to defects in phosphorylation and response to the ligand.

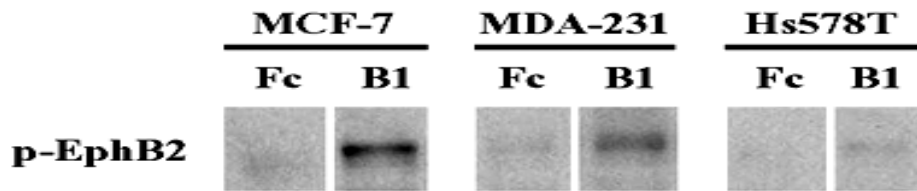


FIGURE 3. Western blot of different breast cancer cell lines treated with a soluble Ephrin-B1-Fc ligand (B1) or the control Fc fragment (Fc), for three hours. Staining was done using an anti-phosphoEphB2 antibody.

In addition to our cell lines models, we are currently assessing tumor biopsies for the differential expression of Ephs/Ephrins, especially EphB2 and its cognate ligands, using immunohistochemistry (data not shown).

Task 2. Eph/Ephrins overexpression or depletion in breast cancer cell lines.

We next designed siRNA sequences for the EphB2 receptor using the pSUPER-Retro retroviral system (Oligoengine inc.). Three sequences were chosen in the EphB2 open reading frame, synthesized and cloned into the pSUPER-Retro vector. Retroviral particles were prepared in Phoenix packaging cells and used to infect three different cell lines which express high levels of EphB2 (MDA-231, BT-20 and Hs578T). Multiple stable clones have been selected and we are in the process of characterizing them for EphB2 silencing levels.

Task 3. Study of interaction between transfected tumor cell lines and endothelial cells *in vitro*.

We are optimizing spheroid formation assays for MDA-231, BT-20, Hs578T breast cancer cell lines and for human embilical endothelial cells (HUVECs available commercially). Spheroids will also be prepared from EphB2 siRNA-transfected cells obtained in task 2. Once optimized, the spheroids will be assessed in breast cancer-endothelial cells co-cultures. Attempts of transendothelial migration assays of tumor cells in a HUVECs layer have been made. However, we are also still optimizing this technique using our cellular models.

Task 4. Metastatic potential of transfected tumor cell lines *in vivo*.

This task has the following experiments.

- a- Subcutaneous and intravenous injection of tumor cells to nude mice.
- b- Tumor growth measurements, bone marrow sampling and screening for metastatic cells by immunocytochemistry using anticytokeratin antibodies

We plan to completely perform these experiments within the next year. Data analysis will be included in our final report.

KEY ACCOMPLISHMENTS

- 1) Poster presentation at the fourth Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), 2005, Philadelphia, PA, USA. Induction of Ephs/Ephrins-mediated tumor cells-endothelial cells repulsion as an anti-cancer therapeutic approach.
- 2) **M. Kandouz**, J. Zhao, I. Oviedo-Landaverde, and G. Batist. The EphB2 Receptor Tyrosine Kinase is a Breast Cancer Tumor Suppressor Gene. *Manuscript in preparation*.

CONCLUSIONS

We have made a significant progress in the realization of this project. We have a pattern of expression of the Eph/Ephrin proteins in the mammary cells and we have a candidate, EphB2, for the proof of concept of our therapeutic strategy. However some aspects needed to be optimized and took more time than initially predicted. This justified our request for a one year no-cost extension of the present award. We will make use of the next year to finalize the remaining experiments and have a conclusion regarding our concept.

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- Sela-Donenfeld D, Wilkinson DG. Eph receptors: two ways to sharpen boundaries. *Curr Biol.* 2005 Mar 29;15(6):R210-2.